RESEARCH ARTICLE

African Invertebrates
A journal of biodiversity research

Molecular phylogeny of *Kazimierzus* Plisko, 2006 (Clitellata, Kazimierzidae) from the Western and Northern Cape Province inferred from mitochondrial DNA sequences

Thembeka Clara Nxele[1,4], Jadwiga Danuta Plisko[1,2], Tarombera Mwabvu[3,4], Oliver Tendayi Zishiri[4]

1 *KwaZulu-Natal Museum, P. Bag 9070, Pietermaritzburg, 3200, South Africa* 2 *School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Pietermaritzburg campus, 3209, South Africa* 3 *School of Biology & Environmental Sciences, University of Mpumalanga, Private Bag X11283, Mbombela, 1200, South Africa* 4 *School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Westville campus, Durban, 4001, South Africa*

Corresponding author: Thembeka C. Nxele (tnxele@nmsa.org.za)

Abstract

Species identification of earthworms using morphology can be challenging and inconclusive as homoplasy in many characters is high. The use of molecular DNA technology, such as the use of conserved regions in mtDNA and nuclear DNA has unravelled the phylogenetic background of several earthworm species. The current study utilised the cytochrome c oxidase subunit I (COI) mitochondrial marker to reconstruct the phylogeny of *Kazimierzus* Plisko, 2006 species from the Western and Northern Cape provinces of South Africa. Phylogenetic reconstructions were implemented using Bayesian Inference, as well as Maximum Likelihood. Both tree building methods adhered to the monophyly of the majority of the taxa. Results showed that species fell into two clades and validated eleven currently known *Kazimierzus* species (*K. circulatus* (Plisko, 1998), *K. franciscus* (Pickford, 1975), *K. guntheri* (Pickford, 1975), *K. hamerae* (Plisko, 1998), *K. kleinoodi* Nxele & Plisko, 2017, *K. nietvoorbiji* Nxele & Plisko, 2017, *K. nieuwoudtvillensis* Nxele & Plisko, 2017, *K. occidualis* (Plisko, 1998), *K. pearsonianus* (Pickford, 1975), *K. phumlani* Nxele & Plisko, 2017, *K. sophieae* (Plisko, 2002)). Cryptic diversity is evident in *K. occidualis* with genetic diver-

gence greater than 12% amongst populations. *Kazimierzus franciscus* and *K. ljungstroemi* (Pickford, 1975) have a low genetic variability suggesting close relatedness or probably conspecificity. A group of specimens from Clanwilliam are morphologically identical to *K. sophieae*, but are genetically distinct and may belong to undescribed species. This study demonstrates the importance of integrative taxonomy in earthworms in order to present reliable taxonomic and biogeographic data.

Keywords
DNA, COI, mtDNA, taxonomy, earthworms, cryptic diversity

Introduction

Earthworms constitute a large component of soil invertebrates and are regarded as soil engineers (Jouquet et al. 2006). They alter soil properties and enhance nutrient cycling (Lavelle 1988) which determines plant community composition. Regardless of their importance, African earthworm fauna have not received much attention and their evolutionary relationships are subject to debate. Species identification is possible by investigating their anatomy (Chang et al. 2007, Csuzdi 2010, Csuzdi and Zicsi 2003, Plisko and Zicsi 1991), but the structural simplicity of their body plan and existence of cryptic species may hinder taxonomic classification (Domínguez et al. 2015). Traditional morphology-based identification also requires substantial taxonomic expertise in this group, because it involves observation of minute morphological characters (Richard et al. 2010). As such, the use of molecular DNA technology in species delineation is an important complement to morphological classification of earthworms.

The use of DNA sequences has increased in the recent past, because it is less subjective than morphological characters, allows for the analysis of several characters (Scotland et al. 2003) and is applicable at all developmental stages (Decaëns et al. 2013; Chang and James 2011). The molecular studies of earthworms that have used the mitochondrial cytochrome c oxidase subunit I (COI) gene in integrative taxonomy have shown good results (Blakemore 2013a, b; Blakemore et al. 2010; Bantaowong et al. 2011; Huang et al. 2007; Richard et al. 2010; King et al. 2008; Rougerie et al. 2009; Chang et al. 2009; James et al. 2010). This marker is able to distinguish the intra- and interspecific genetic variation and groups conspecifics together, because COI sequences are variable enough to differentiate between taxa, but are less variable in conspecifics (Rougerie et al. 2009; Stoeckle and Hebert 2008; Valentini et al. 2008).

According to Nxele (2012), knowledge of the taxonomic diversity of the indigenous megadriles in South Africa is incomplete. Therefore, integration of taxonomic methods is vital to improve the knowledge and understanding of the megadrile fauna. Furthermore, Plisko (2013) stressed that a molecular study on indigenous South African megadriles is essential in order to reveal the evolutionary relationships amongst them. Against this background, the phylogenetic relationships in *Kazimierzus*, a genus occurring in the Western and Northern Cape provinces of South Africa, was investigated.

Materials and methods

Sampling

In order to obtain earthworms of *Kazimierzus*, qualitative sampling was carried out in 2011 and 2015 during the rainy season (July–September) in the Western and Northern Cape, South Africa. Besides the focus on type localities, potential sites other than the type localities were also sampled. Earthworms were collected by digging and hand sorting. Collected specimens were anaesthetised in 20% ethanol solution, fixed in 4% formalin solution and preserved in 75% ethanol for taxonomic purposes. A subsample was preserved in absolute ethanol for molecular analysis. All specimens were examined using a Wild Heerbrugg stereomicroscope and were identified according to the descriptions in Plisko (1996, 1998, 2002), Pickford (1975), Michaelsen (1913) and Nxele et al. (2017). All specimens are at the KwaZulu-Natal Museum, Pietermaritzburg, South Africa.

Genomic DNA extraction, amplification and sequencing

Tissue from the posterior section of the earthworm was used. All DNA extractions were performed using the ZR Genomic DNA™ Tissue MicroPrep kit, following the manufacturer's standard protocol. The concentration of DNA in each sample was estimated using the NanoDrop 2000 (Thermo Scientific). A fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using LCO1490 (5' GGTCAACAAATCATAAAGATATTGG 3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA 3') primers (Folmer et al. 1994). Polymerase chain reactions (PCR) were performed in a final volume of 25 µl using the BIO RAD T100 Thermal Cycler and contained: 2 µl of DNA template (approximately 35 ng/ul), 12.5 µl One Taq Quick-Load 2X Master Mix with standard buffer, 0.5 µl of 10 uM forward and reverse primers and sterile water. The thermocycler conditions were as follows: 95 °C for 2.30 min for initial denaturation, followed by 35 cycles at 95 °C for 30 sec denaturation, 50 °C to 52 °C for 45 sec annealing and 72 °C for 75 sec extension. A final extension step at 72 °C for 10 min completed the reactions.

Sequencing of the 675 bp fragment of the COI mtDNA was conducted at Inqaba Biotechnical Industries (Pty) Ltd.

Sequence alignment and phylogenetic analysis

Identity of sequences was verified by the Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology (NCBI). Sequences of *Amynthas minimus* (Horst, 1893) and *Amynthas corticis* (Kinberg, 1867) were included as outgroup taxa. The sequences for outgroup taxa were obtained from GenBank (Accession nos: AB542509.1, AB542469.1) and current sequences are added as supplementary data.

All specimens are at the KwaZulu-Natal Museum. The sequences were aligned using CLUSTAL X 2.1 (Larkin et al. 2007). These alignments were then manually edited using BIOEDIT 3.3.19.0 (Hall 1998). Unreliable nucleotides (low signal strength), as well as primers sequences, were trimmed off at both the 5' and 3' ends. The programme JMODELTEST v.0.1.1 (Darriba et al. 2012) was used to select the best-fit evolutionary model using the AKAIKE Information Criterion (AIC; Akaike 1973). Phylogenetic analyses were based on two approaches, Bayesian Inference (BI) was performed using MRBAYES 3.2 (Huelsenbeck and Ronquist 2001) and Maximum Likelihood (ML) analysis was performed using GARLI (Zwickl 2011). In each case, the best-fit evolutionary model selected by JMODELTEST was specified.

Clade support was evaluated by 1000 bootstrap replicates for the ML analysis and posterior probability values for the Bayesian analysis. For Bayesian analyses, all MRBAYES analyses were run for 5000000 generations with a sampling frequency of 1000. The deviation of split frequencies was less than 0.01 at the conclusion of all analyses which confirmed that the MCMC chains had converged. The programme TRACER v1.5 (Drummond and Rambaut 2007) was used to check that the Effective Sampling Size > 200 and that posterior distribution for all parameters was unimodal. Consensus trees were generated using PHYLIP 3.69 (Felsenstein 2005) and viewed in FIG TREE v1.3.1 (Rambaut 2009). Uncorrected p genetic distances were obtained for the sequenced specimens using MEGA 6 (Tamura et al. 2013). Each species is represented by one specimen, except where the species appeared in more than one clade on the phylogenetic tree. Sequences have been deposited in GenBank (Table 1).

Results

Phylogenetic analyses

The sequences were 675 bp. Variable sites were 431 bp and conserved sites 233 bp showing great differentiation amongst taxa.

Most species pair comparisons showed a genetic distance above 13%, except for *K. ljungstroemi* and *K. franciscus* which have one percent genetic distance between them (Table 2).

The MI and BI trees were congruent, therefore, support values were annotated on to the branches of the most likely trees generated for each of the datasets analysed (ML run with no bootstrap, rooted using outgroup species (*Amynthas minimus* and *Amynthas corticis*).

Bootstrap values and posterior probabilities below 50% and 0.50, respectively, were not shown on the trees. Two clades, A and B, are distinct; clade A separates further to clades C and D whilst clade B separates to clades E and F (Fig. 1). Both tree building methods support monophyly of the majority of taxa (Fig. 1). *Kazimierzus sophieae* is paraphyletic, found in two distinct clades; one shared with K. sp and the second clade

Table 1. Genbank COI sequences for investigated *Kazimierzus* species.

Species	Specimen catalog #	Genbank #
K. circulatus	NMSA/OLIG.04942/3a,c	MN982467–68
K. franciscus	NMSA/OLIG.09659a,b,c	MN982469–71
K. guntheri	NMSA/OLIG.04986a,b,e	MN982474–75; MN982478
K. hamerae	NMSA/OLIG.04956a,b,c	MN982461–63
K. kleinoodi	NMSA/OLIG.04987	MN982480
K. ljungstroemi	NMSA/OLIG.06960b,d,e	MN982472–73; MN982482
K. nietvoorbiji	NMSA/OLIG.04988a,c	MN982456–57
K. nieuwoudtvillensis	NMSA/OLIG.04990b,c,d	MN982458–60
K. occidualis	NMSA/OLIG.04958a,c,d; NMSA/OLIG.04962	MN982464–66; MN982485
K. pearsonianus	NMSA/OLIG.04984	MN982479
K. phumlani	NMSA/OLIG. 04951/2b,c,e	MN982453–55
K. sophieae	NMSA/OLIG.04950a,c; NMSA/ OLIG.04963a,b	MN982483–84; MN982486–87
K. sp.	NMSA/OLIG.04989a,d,e	MN982476–77; MN982481
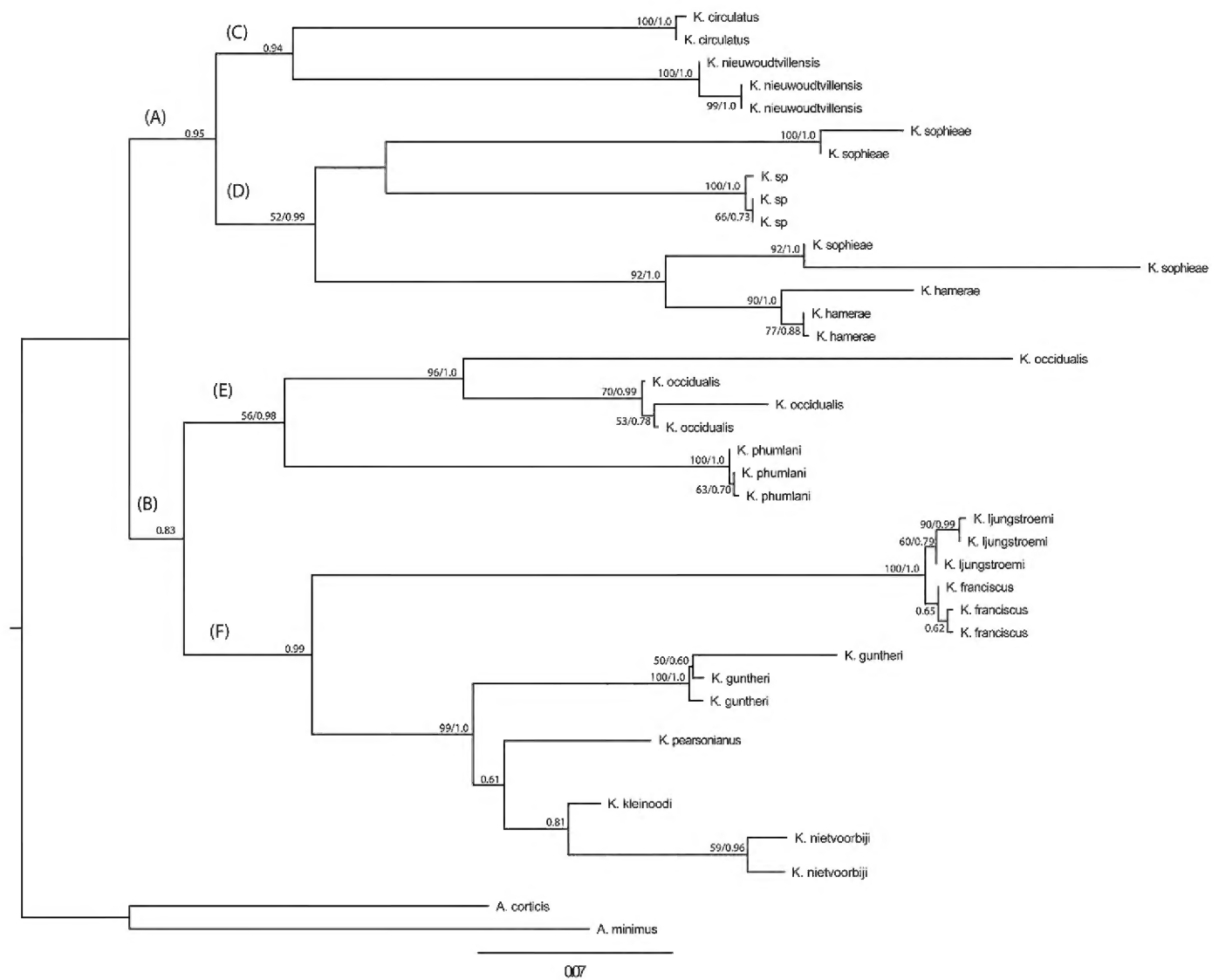
Figure 1. COI gene phylogram showing relationships amongst *Kazimierzus* species. Numbers above nodes are bootstrap support/posterior probabilities from Maximum Likelihood and Bayesian analyses. Letters A–F represents different clades.

Table 2. Pairwise p genetic distances (%) between the investigated *Kazimierzus* species/lineages.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *K. nietvoorbiji*																
2. *K. phumlani*	21.1															
3. *K. hamerae*	22.3	20.1														
4. *K. sophieae*	25.4	25.5	16.9													
5. *K.* sp	20.3	18.3	17.5	23.0												
6. *K. sophieae*	23.1	20.1	21.5	25.5	19.0											
7. *K. nieuwoudtvillensis*	18.8	20.7	19.9	25.5	17.4	22.6										
8. *K. circulatus*	19.0	17.5	18.5	23.8	17.9	19.5	16.6									
9. *K. occidualis*	18.7	15.5	20.9	25.2	18.2	19.6	17.7	15.8								
10. *K. occidualis*	19.0	16.9	22.2	26.3	19.5	20.9	19.0	17.2	3.0							
11. *K. occidualis*	21.5	21.9	23.1	28.5	21.5	24.2	20.4	19.8	15.5	16.1						
12. *K. franciscus*	19.9	19.9	22.0	26.8	19.3	21.7	19.1	19.9	18.7	19.8	23.6					
13. *K. ljungstroemi*	19.9	20.4	22.2	27.3	18.8	21.9	19.3	19.8	18.7	19.6	23.4	1.1				
14. *K. guntheri*	13.6	21.1	21.1	26.6	20.7	23.1	19.3	20.4	19.5	18.7	19.3	21.4	21.5			
15. *K. kleinoodi*	13.2	21.4	23.9	28.5	21.9	23.8	20.9	22.5	19.5	20.7	22.6	22.6	22.8	15.0		
16. *K. pearsonianus*	14.0	22.6	23.8	27.3	23.4	25.4	22.2	22.0	21.7	22.3	23.6	22.2	22.0	15.0	15.8	

with *Kazimierzus hamerae* (clade D). The two species in clade C, *K. circulatus* and *K. nieuwoudtvillensis*, are sister taxa. *Kazimierzus phumlani* and *K. occidualis* are sister taxa comprising clade E. Clade F has six species including a strongly-supported sub-clade, composed of *K. franciscus* and *K. ljungstroemi*. The other species (*K. guntheri, pearsonianus, kleinoodi* and *nietvoorbiji*) form the other sub-clade.

Discussion

Morphological examination revealed eleven currently known species (*circulatus, franciscus, guntheri, hamerae, kleinoodi, nietvoorbiji, nieuwoudtvillensis, occidualis, pearsonianus, phumlani* and *sophieae*). However, the current phylogenetic analysis resulted in additional lineages that suggest cryptic diversity (Fig. 1) and a new species, *K.* sp. Most lineages had strong support but weak branch support was noted in deeper nodes with some clades having bootstrap values less than 50%, hence not annotated on the tree. Genetic distances also support the relationships observed in the phylogenetic tree. Genetic distances between species were all greater than 13% and generally much greater than that, consistent with the recommendations of Hebert et al. (2003), Decaëns et al. (2013) and Huang et al. (2007) for separation of species by genetic distance using the CO1 barcode (Table 2).

Kazimierzus hamerae is similar in appearance to *K. sophieae*, but molecular data confirmed that they are separate species. The phylogenetic tree (Fig. 1), however, highlights, unexpectedly, that *K. sophieae* specimens that were collected from two localities, one in Clanwilliam and the other in van Rhyn's Pass, fall into two clades. The two clades may be because *K. sophieae* is polyphyletic or there is no morphological divergence of these taxa, but are different species genetically (25.5% divergence). The type locality of *K. sophieae* is Nieuwoudtville, which is close to van Rhyn's Pass. It is likely that the

specimens of *K. sophieae* from van Rhyn's Pass that are in the same clade as *K. hamerae* are the real *K. sophieae*. The specimens of *K. sophieae* from Clanwilliam that are in the same clade as *K.* sp. may possibly be an undescribed species; however, a comparison of sequences of *K. sophieae* specimens and those from the type locality, Nieuwoudtville is necessary. It seems that morphological evolution is not rapid in this complex of taxa and there is presence of cryptic diversity. Clade D has poor ML support (52%) but BI support is high (0.99). The low ML support may suggest that the present data are not sufficient to resolve this polytomy fully.

Cryptic diversity was observed in *K. occidualis*. Finding cryptic diversity is common in earthworms, Novo et al. (2010) reporting five cryptic species within the complex of *Hormogaster elisae*. Richard et al. (2010) reported cryptic species in *Lumbricus terrestris* and Pérez-Losada et al. (2009) found cryptic diversity within *Aporrectodea caliginosa* species complex. The observations support the view that diagnosis, based on morphology, only underestimates taxonomic diversity.

The specimens of *Kazimierzus franciscus* and *Kazimierzus ljungstroemi* were collected in two neighbouring forests, Duiwelbos and Koloniesbos in Marloth Nature Reserve, Swellendam. Duiwelbos is the type locality of *K. franciscus*, whilst the type locality for *K. ljungstroemi* is Great Winterhoek, Tulbagh District. Therefore, it is possible that all specimens belong to *K. franciscus*, but it would be of benefit to collect specimens from Winterhoek and compare them with the ones collected from Marloth Nature Reserve.

Although analysis of other conserved genomic regions in both mtDNA and nuclear DNA in the future would benefit the study of *Kazimierzus* species, the phylogenetic analysis of COI recovered several well-supported phylogenetic relationships, some of which are congruent with existing classification.

Acknowledgements

References

Akaike H (1973) Information theory and an extention of the maximum likelihood principle. In: Petrov BN, Csaki F (Eds) Second International Symposium on Information Theory. Akademiai Kiado, Budapest (Hungary), 267–281.

Bantaowong U, Chanabun R, Tongkerd P, Sutcharit C, James SW, Panha S (2011) New earthworm species of the genus Amynthas Kinberg, 1867 from Thailand (Clitellata, Oligochaeta, Megascolecidae). ZooKeys 90: 35–62. https://doi.org/10.3897/zookeys.90.1121

Blakemore RJ (2013a) *Megascolex* (*Perichaeta*) *diffringens* Baird, 1869 and *Pheretima pingi* Stephenson, 1925 types compared to the *Amynthas corticis* (Kinberg, 1867) and *A. carnosus* (Goto & Hatai, 1899) species-groups (Oligochaeta: Megadrilacea: Megascolecidae). Journal of Species Research 2(2): 99–126. https://doi.org/10.12651/JSR.2013.2.2.099

Blakemore RJ (2013b) Survey of Busan Oligochaeta earthworms supported by DNA barcodes. Journal of Species Research 2(2): 127–144. https://doi.org/10.12651/JSR.2013.2.2.127

Blakemore RJ, Kupriyanova EK, Grygier MJ (2010) Neotypification of *Drawida hattamimizu* Hatai, 1930 (Annelida, Oligochaeta, Megadrili, Moniligastridae) as a model linking mtDNA (COI) sequences to an earthworm type, with a response to the 'Can of Worms' theory of cryptic species. ZooKeys 41: 1–29. https://doi.org/10.3897/zookeys.41.374

Chang C, James S (2011) A critique of earthworm molecular phylogenetics. Pedobiologia 54S: S3–S9. https://doi.org/10.1016/j.pedobi.2011.07.015

Chang C, Lin Y, Chen I, Chuang S, Chen J (2007) Taxonomic re-evaluation of the Taiwanese montane earthworm *Amynthas wulinensis* Tsai, Shen &Tsai, 2001 (Oligochaeta: Megascolecidae): polytypic species or species complex? Organisms, Diversity & Evolution 7(3): 231–240. https://doi.org/10.1016/j.ode.2006.06.001

Chang CH, Rougerie R, Chen JH (2009) Identifying earthworms through DNA barcodes: Pitfalls and promise. Pedobiologia 52(3): 171–180. https://doi.org/10.1016/j.pedobi.2008.08.002

Csuzdi C (2010) A monograph of the Paleotropical Benhamiinae earthworms. (Annelida: Oligochaeta, Acanthodrilidae). In: Csudi Cs, Mahunka S (Eds) Pedozoologica Hungarica, Taxonomic, zoogeographic and faunistic studies on soil animals, No 6. Hungarian Natural History Museum, Budapest.

Csuzdi C, Zicsi A (2003) Earthworms of Hungary (Annelida: Oligochaeta, Lumbricidae). In: Csudi CS, Mahunka S (Eds) Pedozoologica Hungarica, Taxonomic, zoogeographic and faunistic studies on soil animals, No 1. Hungarian Natural History Museum, Budapest.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModeltest 2: More models, new heuristics and parallel computing. Nature Methods 9(8): 772. https://doi.org/10.1038/nmeth.2109

Decaëns T, Porco D, Rougerie R, Brown GG, James SW (2013) Potential of DNA barcoding for earthworm research in taxonomy and ecology. Applied Soil Ecology 65: 35–42. https://doi.org/10.1016/j.apsoil.2013.01.001

Domínguez J, Aira M, Breinholt JW, Stojanovic M, James SW, Pérez-Losada M (2015) Underground evolution: New roots for the old tree of lumbricid earthworms. Molecular Phylogenetics and Evolution 83: 7–19. https://doi.org/10.1016/j.ympev.2014.10.024

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7(1): 214. https://doi.org/10.1186/1471-2148-7-214

Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA Primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Hall TA (1998) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41: 95–98.

Hebert PDN, Ratnasingham S, deWaard JR (2003) Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings. Biological Sciences 270(Suppl.): S96–S99. https://doi.org/10.1098/rsbl.2003.0025

Horst R (1893) Earthworms from the Malay Archipelago. In: Weber M (Ed.) Zoologische Ergebnisse einer Reise in Niederländisch OstIndien. 3.E.J. Brill, Leiden, 28–77.

Huang J, Xu Q, Sun ZJ, Tang GL, Su ZY (2007) Identifying earthworms through DNA barcodes. Pedobiologia 51(4): 301–309. https://doi.org/10.1016/j.pedobi.2007.05.003

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics (Oxford, England) 17(8): 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

James SW, Porco D, Decaëns T, Richard B, Rougerie R, Erseus C (2010) DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L., 1758 (Clitellata): Resurrection of *L. herculeus* (Savigny, 1826). PLoS One 5(12): e15629. https://doi.org/10.1371/journal.pone.0015629

Jouquet P, Dauber J, Lagerlöf J, Lavelle P, Lepage M (2006) Soil invertebrates as ecosystem engineers: Intended and accidental effects on soil and feedback loops. Applied Soil Ecology 32(2): 153–164. https://doi.org/10.1016/j.apsoil.2005.07.004

Kinberg JGH (1867) Annulata nova. Öfversigt af Kongliga VetenskapsAkademiens Förhandlingar 23: 97–103.

King RA, Tibble AL, Symonson WOC (2008) Opening a can of worms: Unprecedented sympatric cryptic diversity within British lumbricid earthworms. Molecular Ecology 17(21): 4684–4698. https://doi.org/10.1111/j.1365-294X.2008.03931.x

Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, Mcwilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23(21): 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Lavelle P (1988) Earthworms and the soil system. Biology and Fertility of Soils 6(3): 237–251. https://doi.org/10.1007/BF00260820

Michaelsen W (1913) The Oligochaeta of Natal and Zululand. Annals of the Natal Museum 2(4): 397–457.

Novo M, Almodovar A, Fernandez R, Trigo D, Cosin DJD (2010) Cryptic speciation of hormogastrid earthworms revealed by mitochondrial and nuclear data. Molecular Phylogenetics and Evolution 56(1): 507–512. https://doi.org/10.1016/j.ympev.2010.04.010

Nxele TC (2012) The megadrile fauna (Annelida: Oligochaeta) of Queen Elizabeth Park, South Africa: species composition and distribution within different vegetation types. African Invertebrates 53(2): 543–558. https://doi.org/10.5733/afin.053.0207

Nxele TC, Plisko JD, Mwabvu T, Zishiri OT (2017) Four new earthworm species of *Kazimierzus* Plisko, 2006 (Clitellata, Kazimierzidae). Zootaxa 4353(1): 187–194. https://doi.org/10.11646/zootaxa.4353.1.12

Pérez-Losada M, Ricoy M, Marshall JC, Domínguez J (2009) Phylogenetic assessment of the earthworm Aporrectodea caliginosa species complex (Oligochaeta: Lumbricidae) based

on mitochondrial and nuclear DNA sequences. Molecular Phylogenetics and Evolution 52(2): 293–302. https://doi.org/10.1016/j.ympev.2009.04.003

Pickford GE (1975) Contributions to a study of South African Microchaetinae (Annelida: Oligochaeta). Transactions of the Connecticut Academy of Arts and Sciences 46: 13–76.

Plisko JD (1996) Six new earthworm species of the southern African genus *Proandricus* Plisko, 1992 (Oligochaeta: Microchaetidae). Annals of the Natal Museum 37: 295–307.

Plisko JD (1998) New and little-known species of *Microchaetus* Rapp, 1849, with a key to all species and notes on the significance of certain morphological features (Oligochaeta: Microchaetidae). Annals of the Natal Museum 39: 249–300.

Plisko JD (2002) Three new earthworm species of *Microchaetus* Rapp, 1849, and new data on two earlier known species of this genus (Oligochaeta: Microchaetidae). African Invertebrates 43: 205–214.

Plisko JD (2013) A new family Tritogeniidae for the genera *Tritogenia* and *Michalakus*, earlier accredited to the composite Microchaetidae (Annelida: Oligochaeta). African Invertebrates 54(1): 69–92. https://doi.org/10.5733/afin.054.0107

Plisko JD, Zicsi A (1991) Über neue *Tritogenia*-Arten aus Süd-Afrika (Oligochaeta: Microchaetidae). Mitteilungen aus dem Naturhistorischen Museum in Hamburg 88: 111–123.

Rambaut A (2009). Fig Tree. http://tree.bio.ed.ac.uk/software/figtree/

Richard B, Decaëns T, Rougerie R, James SW, Porco D, Hebert PD (2010) Re-integrating earthworm juveniles into soil biodiversity studies: Species identification through DNA barcoding. Molecular Ecology Resources 10(4): 606–614. https://doi.org/10.1111/j.1755-0998.2009.02822.x

Rougerie R, Decaëns T, Deharveng L, Porco D, James SW, Chang C, Richard D, Potapov M, Suhardjono Y, Hebert PDN (2009) DNA barcodes for soil animal taxonomy. Pesquisa Agropecuária Brasileira 44(8): 789–802. https://doi.org/10.1590/S0100-204X2009000800002

Scotland RW, Olmstead RG, Bennet JR (2003) Phylogeny reconstruction: The role of morphology. Systematic Biology 52(4): 539–548. https://doi.org/10.1080/10635150309309

Stoeckle MY, Hebert PDN (2008) Barcode of Life: DNA tags help classify animals. Scientific American 298: 39–43.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Valentini A, Pompanon F, Taberlet P (2008) DNA barcoding for ecologists. Trends in Ecology & Evolution 24(2): 110–117. https://doi.org/10.1016/j.tree.2008.09.011

Zwickl DJ (2011) Garli. http://www.nescent.org/wg/garli